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STANDARD BIOASSAY USING THE BARNACLE LARVA

DAVID W. TAYLOR NAVAL SHIP RESEARCH AND
DEVELOPMENT CENTER, ANNAPOLIS, MARYLAND

FEBRUARY 1977

Report MAT-76-76

Standard Bioassay Using the Barnacle Larva

DAVID W. TAYLOR NAVAL SHIP RESEARCH AND DEVELOPMENT CENTER

Bethesda, Md. 20084

STANDARD BIOASSAY USING THE BARNACLE LARVA

by

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and
Joyce M. Raines

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MATERIALS DEPARTMENT
Annapolis
RESEARCH AND DEVELOPMENT REPORT

February 1977

Report MAT-76-76

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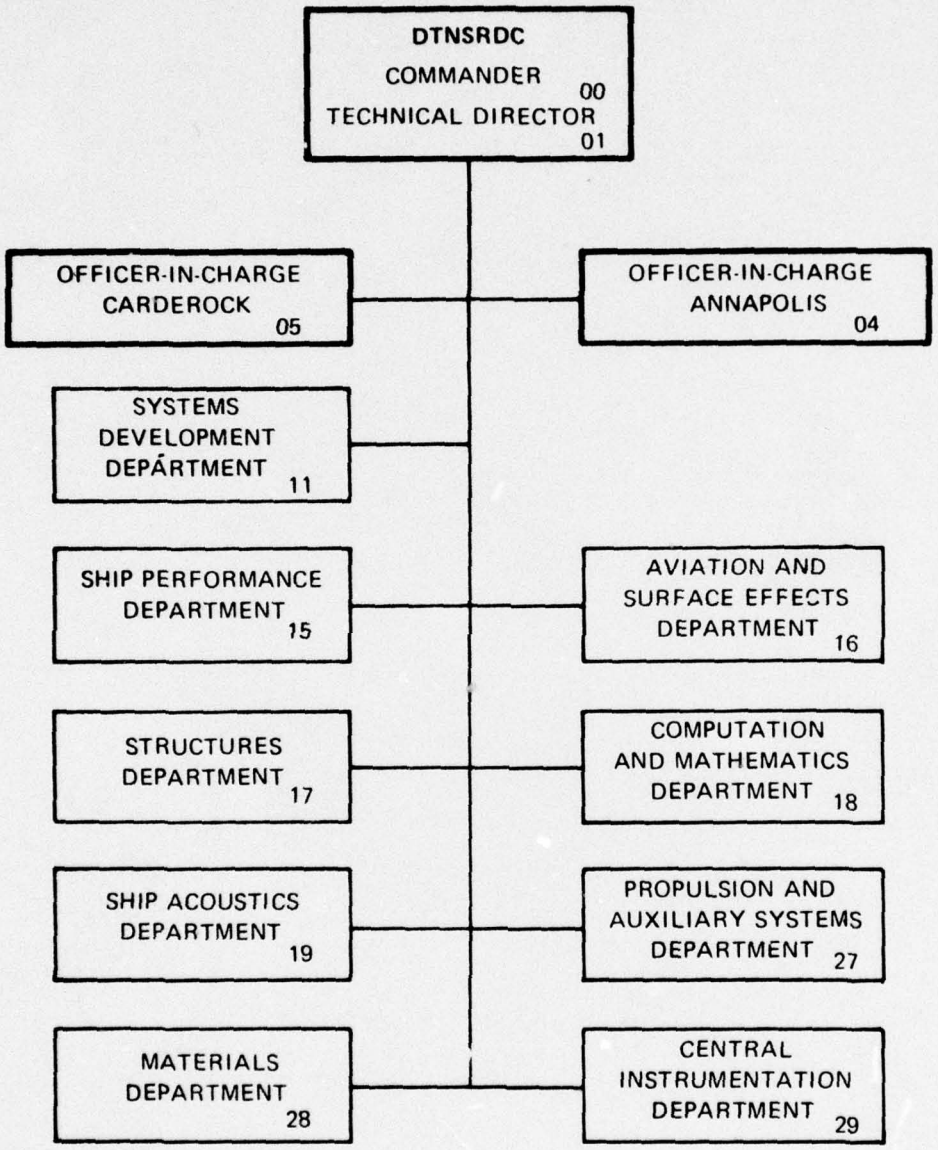

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A technique is developed for the use of the barnacle larva (Balanus amphitrite) as a bioassay organism. Because of its availability, ease of handling, sensitivity, and response, the barnacle larvae are ideal organisms for the determination of the LC_{50} (lethal concentration to 50% of the test population).

(Authors)

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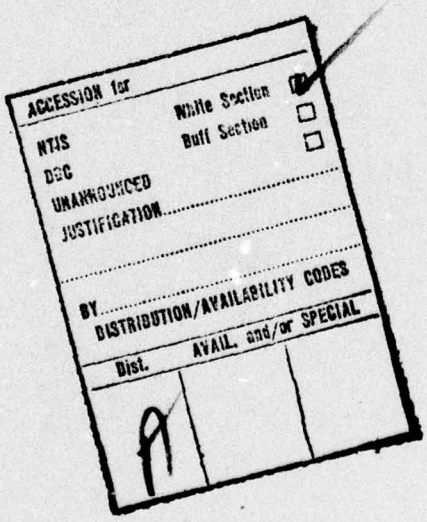
LIST OF ABBREVIATIONS AND SYMBOLS

B. amphitrite	- Balanus amphitrite
° C	- degrees Celsius
CL	- confidence limits
etc	- and so forth
i.e.	- that is
LC_{50}	- lethal concentration to 50% of the test population
ml	- millilitres
mm	- millimetres
µm	- micrometres
TBTO	- tri-butyl tin oxide
‰	- parts per thousand



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ABSTRACT

A technique is developed for the use of the barnacle larva (Balanus amphitrite) as a bioassay organism. Because of its availability, ease of handling, sensitivity, and response, the barnacle larvae are ideal organisms for the determination of the LC_{50} (lethal concentration to 50% of the test population).

INTRODUCTION

The purpose of using barnacle larvae as bioassay organisms is to determine the LC_{50}* (lethal concentration of the sample chemical in the solution which kills half of the total population exposed to it). The LC_{50} value can be accurately determined in a short time due to the large quantities of larvae that are utilized at each test concentration. Because larvae are more sensitive than the adult stage to chemical insult, the experimental test time can be shortened.

This experiment develops sufficient data to prove the "a priori" assumption that the barnacle larva possesses the necessary prerequisites for an excellent bioassay organism. The necessary characteristics of a desirable bioassay organism include: (1) minimal size which permits ease of handling of a large population within a restricted space, (2) availability in large numbers to satisfy statistical requirements, (3) ready availability the entire year and within financial limits, (4) maintenance in chemically reproducible medium (artificial seawater), (5) a specific species being consistently available from a single source, and (6) ease of transportation to allow continued viability from the field (collection site) to the laboratory. The barnacle larva meets the above requirements as follows:

- The test larvae are a combination of the initial three naupliar stages of development, average size ranging from 100-300 μm.

- Barnacles are collected on accretion panels which have many gravid adults, and each gravid adult yields hundreds of larvae.

- The field collection site is in tropical water where the barnacles reproduce the whole year and settling is relatively constant. Cost for exposing the panels and shipping them via air cargo is nominal.

*Definitions of abbreviations and symbols used are on page i.

- Instant Ocean,® a commercially available synthetic sea salt mix (Aquarium Systems, Inc.), is used as the diluent in the experimental runs.

- Each accretion panel is covered with many barnacle species. Balanus amphitrite will attach throughout the entire year.

- The employment of proper packing and shipping procedures insures barnacle viability for a reasonable period of time out of water. The attached adults arrive from the barnacle accretion site within 24 hours after removal from the water.

For the above stated reasons and its wide geographical distribution, the barnacle larva yields significant and useful LC_{50} values.

MATERIALS AND METHODS

The barnacle larvae bioassay method is an adaptation of the original bioassay technique developed in this laboratory.[1] The procedures involved in the experimental runs consists of (1) obtaining the gravid adult barnacles, (2) hatching and collecting the test larvae, (3) preparing the chemicals to be tested, and (4) performing the actual test wherein the test organisms are placed in the test solutions for 24 hours and the LC_{50} is calculated.

ADULT BARNACLES

Adult barnacles are obtained from Miami Marine Research, Inc., Miami, Florida, where accretion panels are suspended 1 metre below the water's surface from floating rafts. When barnacles are needed, the panels are collected, wrapped in wet newspaper, placed in a carton, and shipped via Air Cargo to this Center. (figure 1). The panels arrive at the laboratory within 24 hours after having been pulled out of the water. Upon arrival, they are rinsed with Chesapeake Bay water while being scrubbed with a stiff brush to remove excess fouling materials, thus leaving only the barnacles. The larger barnacles are then speciated by shell configurations. From the species available, Balanus amphitrite is chosen for the test (smooth shell with pink vertical striations) and then pried off the panel.

[1] Superscripts refer to similarly numbered entries in the Technical References at the end of the text.

Figure 1 - Aluminum Accretion Panel

Upon removal, the basal plate of this animal often adheres to the surface of the accretion panel (especially on metal panels). However, if the animal comes off intact, the basal plate must be broken and removed to expose the mantle membrane (figure 2). After removal of the basal plate and with the aid of a dissection microscope, the mantle membrane (with either the ovary and eggs or testes and semen) is gently cut and pushed aside with a specially designed glass needle (figure 3), thus exposing the mantle cavity and the animal's body. If the animal is gravid, two masses of embryos (lamellae) are present in the mantle cavity, one on either side. The gelatinous lamellae are then carefully removed with a glass loop to prevent rupturing the lamellae.

1. Orifice of shell
2. Lateral compartment of shell
3. Scutum
4. Rostrum or ventral compartment of shell
5. Sheath of rostrum
6. Adductor muscle
7. Cut surface of attachment to the removed part of scutum
8. Muscles
9. Opercular membrane
10. Rostral depressor muscle of scutum
11. Oviduct
12. Ovary
13. Antennules
14. Tergum
15. Carina, or dorsal compartment of shell
16. Sheath of carina
17. lst., 2nd., and 3rd. cirri
18. Labrum (anterior part of mouth)
19. Basal articulation of lst. cirrus
20. Aperture of acoustic sac
21. Thorax
22. Carinal depressor muscle of tergum
23. Mantle membrane
24. Basal plate
25. Mantle cavity
26. Prosoma

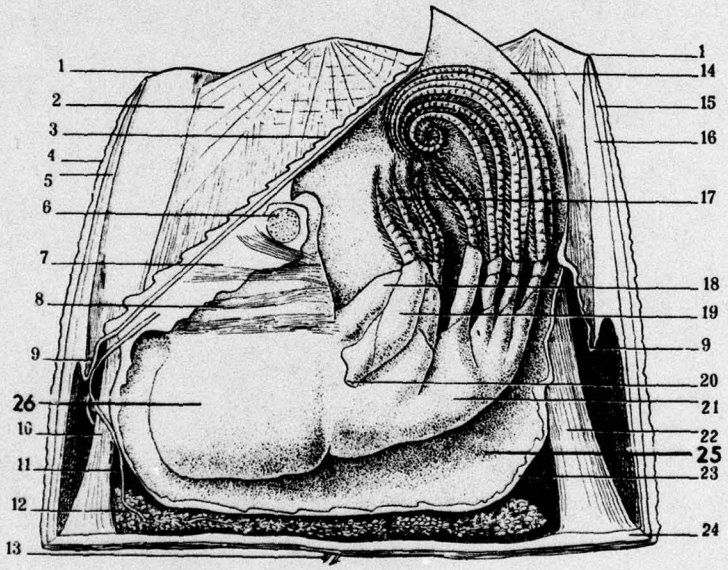

Figure 2 - Barnacle Anatomy

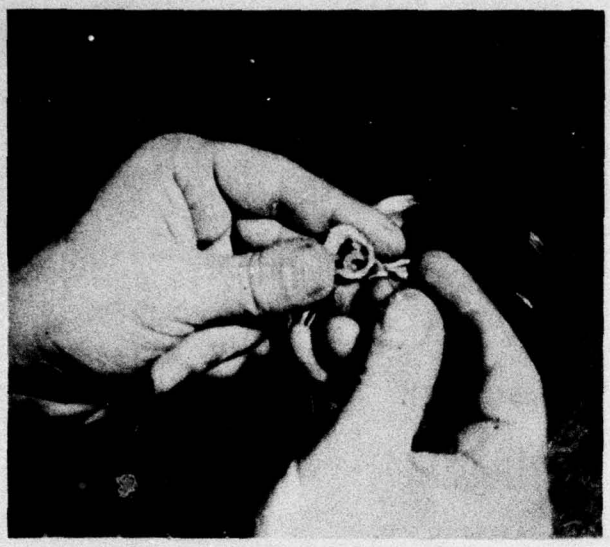

Figure 3
Lamella Extraction

HATCHING AND COLLECTION

All of the lamellae from the B. amphitrite adults are collected in a Pyrex dish containing Instant Ocean. Next, they are segregated into dishes according to state of embryonic development as follows: (1) white - very immature; hatching will occur in 1 week, or it will not occur at all; (2) yellow - immature but the embryos are developing and hatching will occur within 1 week; (3) brown - mature; hatching can occur anytime within 30 minutes to a few days, depending upon degree of maturity, i.e. - brown without eye spots - hatching in a few days; brown with eye spots - hatching in about 1 day; brown with eye spots and motion - hatching will occur in about an hour. Once the lamellae are segregated, they are washed by transferring them to another dish containing Instant Ocean. They are then observed under the microscope for a final check of their condition (physical - intact, broken, etc.; other - fungi, protozoa, and other organisms present in the gelatinous material). If the lamellae are satisfactory, they are finally transferred to hatching vessels and covered. The hatching vessels are small glass beakers (500 ml) with one lamella per 200 ml of Instant Ocean, and all are maintained in an incubator at 20 ±0.5° C.

The collection process begins once hatching has occurred. A light beam is aimed on one side of the hatching beaker, close to the surface. The positively phototropic nauplii swim toward the light[8] and congregate in that area (figure 4). They are immediately drawn into a glass pipette and placed in another dish with filtered Instant Ocean. When sufficient nauplii are collected, the hatching vessels are returned to the incubator. The collected nauplii are washed one or more times by transferring them to other dishes with clean water. After the last

wash, the nauplii are picked up 10 at a time, with minimal fluid carry-over (less than 1 ml) and placed in the test solutions. The dishes with the test solutions (10 ml/dish) and the nauplii (10/dish) are observed for 24 hours and are maintained under constant illumination in the incubator.

Figure 4 - Naupliar Collection

TEST CHEMICAL PREPARATION

All of the dilutions are made with Instant Ocean (salinity 35‰ and pH 7.8). About 20 litres of the artificial seawater are prepared for each new shipment of barnacles, thus ensuring fresh or newly mixed artificial seawater as the diluent.

The plastic test equipment (i.e., Petri dishes, pipettes, etc) are used once, then discarded. The glass pipettes are washed with detergent, rinsed four or five times with tap water, three or four times with distilled water, and two times with ethanol after each use.

Solutions are made on the basis of: (1) percent of the original stock (if original test chemical is either not known or is a mix) or (2) parts per million or billion (if the original chemical concentration is known).

ACTUAL TEST RUN

Before adding the nauplii to the test solutions, they are examined under the microscope for: (1) number of larvae present, (2) determination of the vigor, and (3) developmental stage of the nauplii. No attempt is made to separate the three early stages (except to note which is predominant). From this second wash dish, the nauplii are put into the test solutions: ten in each dish or one nauplius for each millilitre of test solution. The 24-hour experimental run then begins. Immediate effects on the nauplii are recorded, and periodic observations are made as often as possible within the test period. The dead organisms are removed at each count. At the 24-hour limit, final counts are taken, and final dead-live numbers are tabulated. Table 1 shows the two essential experimental determinations which are carried out for all chemicals.

TABLE 1
PROCEDURE - TBTO*

First Run		Second Run
1. Control	- 1 dead, 9 alive	1. Control
2. 0.01%	- 3 dead, 7 alive	2. 0.1%
3. 0.1%	- 3 dead, 7 alive	3. 0.2%
4. 1%	- 7 dead, 3 alive	4. 0.3%
5. 10%	- 9 dead, 1 alive	5. 0.4%
		6. 0.5%
		7. 0.6%
		8. 0.7%
		9. 0.8%
		10. 0.9%
		11. 1.0%

*LC_{50} determination is of an original stock solution.
Note: For results see table 2.

The aim of the first trial run is to bracket the limits of toxicity for 50% of the organisms for 24 hours. In the example given in table 1, the results of the first run with a solution of tri-butyl tin oxide indicated that the LC_{50} would be between 0.1% and 1% of the original stock TBTO solution. The second run was then set up to further refine this figure. If necessary, a third test run can be set up for verification or further detailing.

The numbers obtained from the final run are entered in the bioassay data sheet (table 2), and the 24-hour LC_{50} is calculated. This number can either be calculated in percent of the original stock or in parts per million or billion (if the original stock is known). All test runs are conducted at 20 ±0.5° C.

TABLE 2

DAVID W. TAYLOR NAVAL SHIP R & D CENTER
Code 2853
Annapolis, Md. 21402
(301) 267-3354/3535

BIOASSAY DATA SHEET

Bioassay of: TBTO With: Barnacle Larva

Investigator: _____ Ext: _____ Initials: _____ Date: _____

I TEST SAMPLE

1. Sample title and/or #: TBTO/Water Code #: _____
2. Date received: _____ Completed: _____
3. Source: _____
4. Composition and properties: _____

II TEST MEDIUM (DILUENT)

1. Water, Type and/or source: Instant Ocean
2. Salinity: 35 ‰ pH: 7.85
3. Other: filtered

III TEST ORGANISM

1. Species: Balanus amphitrite
2. Age and/or larval stage: First 3 stages (nauplii)
3. Other: Not fed, washed two times prior to testing

IV TEST DESIGN

1. Test vessel, type and volume: Plastic dish, 60 X 15 mm, 10 ml
2. Organisms/vessel: 10 Organism/ML: 1
3. Physical conditions:
   Temp.(°C): 20 ±0.2 Light: Constant illumination
4. Parameter(s) measured: Mortality
5. Other: _____

V TEST RESULTS

1. LC_{50}: 0.71 ±0.13% (95% CL)* Test duration: 24 hours
2. Other: *LC_{50} determination is of an original stock solution.
3. Graph appended: _____

TABLE 2 - (Cont)

Bioassay of: TBTO With: Barnacle Larvae

Method: Computation of LC_{50} by method of Reed and Muench. Davis et al; MICROBIOLOGY, Harper & Row, New York, 1967.

	CONC.	DEAD	ALIVE	TOTAL DEAD ↓	TOTAL ALIVE ↑	TD/TD+TA	MORTALITY
1)	Control	1	9	1	75	1/76	1.32%
2)	0.1%	1	9	2	66	2/68	2.94%
3)	0.2%	1	9	3	57	3/60	5.00%
4)	0.3%	0	10	3	48	3/51	6.00%
5)	0.4%	4	6	7	38	7/45	15.55%
6)	0.5%	2	8	9	32	9/41	21.95%
7)	0.6%	3	7	12	24	12/36	33.33%
8)	0.7%	4	6	16	17	16/33	48.48%
9)	0.8%	5	5	21	11	21/32	65.63%
10)	0.9%	5	5	26	6	26/32	81.25%
11)	1.0%	9	1	35	1	35/36	97.22%
12)							

COMPUTATION OF LC_{50}, HOURS (24)

1. $$\frac{(\text{Higher \% mortality } 65.63) - (50)}{(\text{Higher \% mortality } 65.63) - (\text{Lower \% mortality } 48.48)} = (0.911)\ \text{Prop. factor}$$

2. (Prop. factor 0.911) × (Increment 0.1) = (Increase 0.091)

3. LC_{50} = (Higher conc. 0.8) - (Increase 0.091)

LC_{50} = (24 hours) = 0.71 ± 0.13% (95% confidence limits)*

Observations and/or comments:

Linear regression analysis gives 95% confidence limits of ±0.13.

*LC_{50} determination is of an original stock solution.

DISCUSSION AND CONCLUSIONS

Historically, in medical entomology, the larval stages of an insect have proven to be the most sensitive phases of the life cycle. Hence, those stages have been affected with chemical agents at very low concentrations which probably would be innocuous to the adult. This phenomenon has been found in many unrelated organisms. Due to the rapid increases of metabolism preceding and during larval metamorphoses and the rapid, almost spastic mobility, the larva of the barnacle is very susceptible to chemical insult of a very low magnitude. Because the barnacle larva is "naked to the environment," i.e., more exposed to the environment compared to the adult, it is more accessible to chemical insult than the parent. Since the barnacle larva is often the major contributor to the zooplanktonic base of the food chain in any one ecological niche and because it often summates the local environmental conditions, it is an ideal bioassay organism.

All testing with barnacle larvae must be done only with the first three stages. From hatching to stage three, the time elapsed is approximately 2 to 3 days (see figure 5). The naupliar population being tested is always a mixture of the initial three stages. At these stages, the organisms are relatively active and are still utilizing the yolk as a food source; thus, they do not have to be fed. This eliminates the introduction of a major unknown in the experimental run. Due to the deletion of extra food and extra handling, and the constant temperature and salinity of the solutions, the survival in the control dishes is consistently over 90%. If this survival rate drops near 80% or below (in the control), the run is discarded and started de novo, since this would indicate trouble with the lamella, the organism, or the hatching process. The organisms do not survive for any length of time beyond 2 or 3 days after hatching. This is the fundamental reason why the experimental run is 24 hours in duration. For the runs to be continued after this time period, the nauplii would have to be fed, but such feeding does not always assure a high percentage of survival or a rapid molting to the successive stages. The fourth and fifth stages are much larger and show definite anatomical and physiological differences from the earlier stages (see figure 5). The sixth stage nauplius is preparing to molt to the cyprid larva and starts internal reorganizations to prepare for becoming an entirely different organism. The cyprid is not a useful bioassay organism. The cyprid has one basic function, i.e., attachment. It is adapted only for this final purpose; thus, it is impervious to a host of stimuli. A cyprid does not feed. When it wishes, it can close its bivalve shell for some time, open it on one end, and with very vigorous pushing of its thoracic appendages, it can dart about very fast.

BALANUS AMPHITRITE
LARVAL STAGES

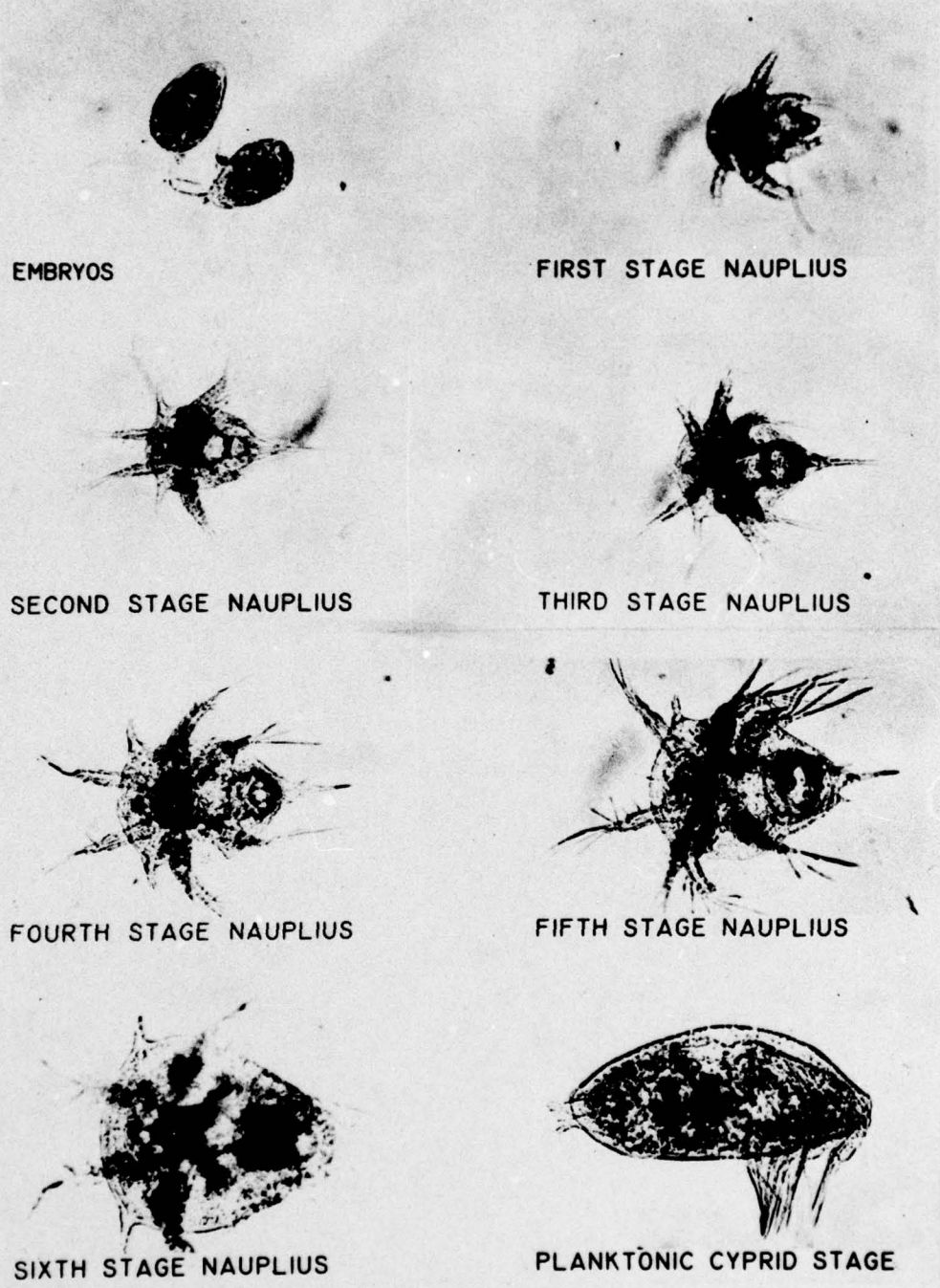

Figure 5 - Larval Stages

Naupliar positive phototropism for collection has many advantages: (1) it offers little mechanical damage to the nauplii; (2) it allows only the most viable nauplii to swim toward the light; (3) it can be done with minimal time and equipment; (4) it is done under microscopic observation, thus allowing close examination of individual nauplii; (5) large numbers can be handled simultaneously, and (6) the correct stage can either be chosen or noted. The adult barnacles (attached on panels) are wet when shipped to our laboratory, but not immersed in water. The animals have been subjected to much stress (removed from the water, shipped, etc). If they were to be returned to water, most of them would release the embryos. This is the common biological phenomenon; reproduction in adversity.[3]

Experiments under way at DTNSRDC indicate that, in addition to high sensitivity to many chemicals (which will be the basis for a future report), this bioassay procedure is: (1) less time consuming, 2 days compared to the normally accepted time (8 days) for complete assay of adult organisms; (2) less space consuming, the space needed is that which holds 15-20 small dishes (60 x 15 mm) compared to fish tanks or other large vessels; (3) less money consuming, gravid barnacles can be obtained for a nominal fee and the needed equipment is readily available within any laboratory; and (4) less variable, the organism is ubiquitous and indigenous to many geographic locations, and the experimental runs are conducted in a chemically reproducible medium (artificial seawater).

TECHNICAL REFERENCES

1 - Liberatore, G., "DTNSRDC Standard Static Marine Bioassay Procedure for Shipboard Chemicals," DTNSRDC Tech Memo 28-76-29 (1976)

2 - "A Review of Barnacle Photosensitivity and Its Possible Use for Marine Fouling Control," USNASL Lab Project IED-12, Tech Memo 3, AD840-409 (1968)

3 - Sumich, J. L., "An Introduction to the Biology of Marine Life," Dubuque, Iowa, Wm. C. Brown Company (1976)